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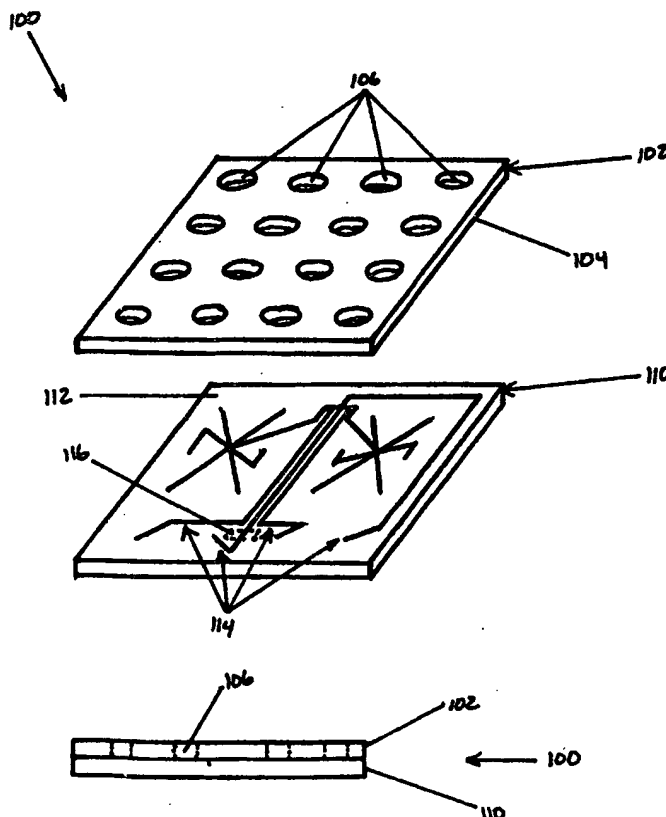
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(54) Title: MICROFLUIDIC DEVICES AND SYSTEMS INCORPORATING COVER LAYERS

(57) Abstract

The present invention provides microfluidic devices that comprise a body structure (100) comprising at least a first microscale channel network (114) disposed therein. The body structure (100) has a plurality of ports (106) disposed in the body structure (100), where each port (106) is in fluid communication with one or more channels (114) in the first channel network. The devices also include a cover layer (200) comprising a plurality of apertures (206) disposed through the cover layer (200). The cover layer (200) is mated with the body structure (100) whereby each of the apertures (206) is aligned with a separate one of the plurality of ports (106).



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5 MICROFLUIDIC DEVICES AND SYSTEMS INCORPORATING COVER LAYERS

10 BACKGROUND OF THE INVENTION

 As has been the case in the electronics and computer industries, trends in analytical chemical and biochemical instrumentation have been toward miniaturization. In chemical and biochemical analyses, such miniaturization as achieved in e.g., microfluidic systems, provides numerous advantages, including significantly smaller reagent requirements, faster throughput, ready automatability, and in many cases, improved data.

 By way of example, U.S. Patent Nos. 5,498,392 and 5,587,128 describe the performance of amplification reactions in microfabricated devices including microscale flow systems and/or reaction chambers. Such systems substantially reduce the requirements for expensive reagents utilized in amplification reactions. Further, the small scale of these devices also provides for enhanced thermal transfer between heating sources and the reagents in the device.

 Similarly, U.S. Patent No. 5,637,469 describes the use of devices having extremely small internal dimensions for detecting an analyte in a sample via a binding assay. Again, the small scale of such devices provides advantages in terms of small reagent volumes.

 Commonly owned Published International Application No. WO 98/00231 describes the use of microfluidic devices and systems in the performance of high-throughput screening assays. Again, these systems reduce the required volumes of potentially very expensive test compounds, e.g., drug candidates, library compounds, etc.

 Despite the numerous advantages realized by the miniaturization of analytical systems, such miniaturization can provide difficulties in the use of such systems, including user handling and system interfacing of such devices.

 It would therefore be desirable to provide microfluidic devices that capture the advantages associated with extremely small volumes and dimensions, without the

problems associated with such small-scale devices. The present invention meets these and a variety of other needs.

SUMMARY OF THE INVENTION

It is a general object of the present invention to provide microfluidic devices that combine the advantages of microfluidics with improved material handling characteristics and reduced costs for manufacturing. The present invention accomplishes this in a first aspect, by providing microfluidic devices that incorporate a body structure comprising at least a first microscale channel network disposed therein. The body structure has a plurality of ports disposed in it, where each port is in fluid communication with one or more channels in the first channel network. The devices also include a cover layer comprising a plurality of apertures disposed therethrough. The cover layer is mated with the body structure whereby each of the apertures is aligned with a separate one of the plurality of ports.

In preferred aspects, each of the body structure and the cover layer separately comprises at least a first surface. The plurality of ports in the body structure are disposed in the first surface of the body structure, and the plurality of apertures in the cover layer are disposed in the first surface of the cover layer. The first surface of the cover layer is mated to the first surface of the body structure such that the apertures align with and are in fluid communication with the ports. In further preferred aspects, the cover layer is fabricated from a polymeric material, and is preferably an injection molded polymeric part.

In a related aspect, the present invention provides a microfluidic system that includes a microfluidic device in accordance with the present invention, where the device is further mounted on a controller/detector apparatus that is configured to receive the microfluidic device. The controller/detector apparatus comprises an optical detection system and a material transport system, where the detection system and transport system are operably interfaced with the microfluidic device when the device is mounted on the controller/detector.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 schematically illustrates a microfluidic device body structure that incorporates a planar layered structure.

Figures 2A-E illustrate from a number of perspectives an embodiment of a cover layer for incorporation in a microfluidic device in accordance with the present invention.

Figure 2F illustrates the interaction of a filling device with the microfluidic devices of the invention.

Figure 3A illustrates a fully assembled microfluidic device that includes the layered body structure of Figure 1 and the cover layer of Figure 2 mated together. Figure 3B illustrates an alternate mechanism for joining the body structure to the cover layer in the fully assembled device.

DETAILED DESCRIPTION OF THE INVENTION

I. General

The present invention generally provides microfluidic devices that take advantage of the extremely small-scale nature of microfluidic devices and systems, while at the same time, not suffering from some of the potential problems associated with such systems. In particular, the microfluidic devices and systems of the invention include an additional cover layer as a portion of the microfluidic device, e.g., overlaying and attached to the basic body structure of the device. The cover layer employed in the devices of the invention typically comprises a number of apertures disposed through it, which apertures mate with and/or form part of the reservoirs and/or access ports of the microfluidic device. These cover layers provide a number of advantages in the operation and fabrication of microfluidic devices.

As used herein, the terms "microscale," "microfabricated" or "microfluidic" generally refer to one or more fluid passages, chambers or conduits which have at least one internal cross-sectional dimension, e.g., depth, width, length, diameter, etc., that is less than 500 μm , and typically between about 0.1 μm and about 500 μm . In the devices of the present invention, the microscale channels or chambers preferably have at least one cross-sectional dimension between about 0.1 μm and 200 μm , more preferably between about 0.1 μm and 100 μm , and often between about 0.1 μm and 20 μm . Accordingly, the microfluidic devices or systems prepared in accordance with the present invention typically include at least one microscale channel, usually at least two intersecting microscale channels, and often, three or more intersecting channels disposed within a single body structure. Channel intersections may exist in a number of formats, including cross intersections, "T" intersections, or any number of other structures whereby at least two channels are in fluid communication.

The body structure of the microfluidic devices described herein can take a variety of shapes and/or conformations, provided the body structure includes at least one

microfluidic channel element disposed within it. For example, in some cases the body structure has a tubular conformation, e.g., as in capillary structures, such as fused silica or polymeric capillaries that include internal diameters in the microscale range, set forth above. Alternatively, body structures may incorporate non-uniform shapes and/or conformations, depending upon the application for which the device is to be used. In preferred aspects, the body structure of the microfluidic devices incorporates a planar or "chip" structure.

Although in some cases, a single piece body structure, e.g., a capillary, may be used, the devices described herein typically comprise an aggregation of two or more separate layers which when appropriately mated or joined together, form the body structure of the microfluidic device of the invention, e.g., containing the channels and/or chambers described herein. Typically, the microfluidic devices described herein will comprise a top portion, a bottom portion, and an interior portion, wherein the interior portion substantially defines the channels and chambers of the device.

Figure 1 illustrates one example of the body structure of a microfluidic device that incorporates a planar, layered structure. As shown, the body structure 100 includes at least two layers, an upper layer 102 and a lower layer 110. The upper surface 112 of the lower layer 110 is fabricated to include grooves and/or wells 114. The lower surface 104 of the upper layer 102 is then mated to the upper surface 112 of the lower layer 110 such that the grooves and/or channels define channels or conduits, and chambers within the interior of the aggregate body structure.

A variety of substrate materials may be employed as the bottom portion. Typically, because the devices are microfabricated, substrate materials will be selected based upon their compatibility with known microfabrication techniques, e.g., photolithography, wet chemical etching, laser ablation, reactive ion etching (RIE), air abrasion techniques, injection molding, LIGA methods, metal electroforming, embossing, and other techniques. Suitable substrate materials are also generally selected for their compatibility with the full range of conditions to which the microfluidic devices may be exposed, including extremes of pH, temperature, salt concentration, and application of electric fields. Accordingly, in some preferred aspects, the substrate material may include materials normally associated with the semiconductor industry in which such microfabrication techniques are regularly employed, including, e.g., silica based substrates, such as glass, quartz, silicon or polysilicon, as well as other substrate materials, such as gallium arsenide and the like. In the case of semiconductive materials, it will often be desirable to provide an insulating coating or layer, e.g., silicon oxide, over the substrate material, and particularly in those applications where

electric fields are to be applied to the device or its contents. In preferred aspects, the substrates used to fabricate the body structure are silica-based, and more preferably glass or quartz, due to their inertness to the conditions described above, as well as the ease with which they are microfabricated.

5 In alternate preferred aspects, the substrate materials comprise polymeric materials, e.g., plastics, such as polymethylmethacrylate (PMMA), polycarbonate, polytetrafluoroethylene (TEFLON™), polyvinylchloride (PVC), polydimethylsiloxane (PDMS), polysulfone, polystyrene, polymethylpentene, polypropylene, polyethylene, polyvinylidene fluoride, ABS (acrylonitrile-butadiene-styrene copolymer), and the like. Such
10 polymeric substrates are readily manufactured using available microfabrication techniques, as described above, or from microfabricated masters, using well known molding techniques, such as injection molding, embossing or stamping, or by polymerizing the polymeric precursor material within the mold (See U.S. Patent No. 5,512,131). Again, such polymeric substrate materials are preferred for their ease of manufacture, low cost and disposability, as
15 well as their general inertness to most extreme reaction conditions. Again, these polymeric materials may include treated surfaces, e.g., derivatized or coated surfaces, to enhance their utility in the microfluidic system, e.g., provide enhanced fluid direction, e.g., as described in U.S. Patent Application Serial No. 08/843,212, filed April 14, 1997 (Attorney Docket No. 17646-002610), and which is incorporated herein by reference in its entirety for all purposes.

20 In the embodiment shown, the upper layer 102 of the body structure 100, includes a plurality of ports 106 disposed through it. These ports are positioned to communicate with specific points of the channels or grooves 114, e.g., the termini, in the aggregate body structure when the upper and lower layers are mated. The ports 106 function to provide fluid access to the channels of the device, and in certain aspects, electrical access to
25 the channels within the body structure.

In many embodiments, the microfluidic devices include an optical detection window 116 disposed across one or more channels and/or chambers of the device. Optical detection windows are typically transparent such that they are capable of transmitting an optical signal from the channel/chamber over which they are disposed. Optical detection
30 windows may merely be a region of a transparent layer of the body structure, e.g., where the layer is glass or quartz, or a transparent polymer material, e.g., PMMA, polycarbonate, etc. Alternatively, where opaque substrates are used in manufacturing the devices, transparent detection windows fabricated from the above materials may be separately manufactured into the device.

Microfluidic devices may be used in a variety of applications, including, e.g., the performance of high throughput screening assays in drug discovery, immunoassays, diagnostics, genetic analysis, and the like. As such, the devices described herein, will often include multiple sample introduction ports or reservoirs, for the parallel or serial
5 introduction and analysis of multiple samples. Alternatively, these devices may be coupled to a sample introduction port, e.g., a pipettor, which serially introduces multiple samples into the device for analysis. Examples of such sample introduction systems are described in e.g., U.S. Patent Application Nos. 08/761,575 and 08/760,446 (Attorney Docket Nos. 17646-000410 and 17646-000510, respectively) each of which was filed on December 6,
10 1996, and is hereby incorporated by reference in its entirety for all purposes.

In preferred aspects, the microfluidic devices of the present invention utilize electrokinetic material transport systems to direct and transport materials through the channels of the device. As used herein, "electrokinetic material transport" generally refers to systems and methods for transporting and directing materials within an interconnected
15 channel and/or chamber containing structure, through the application of electrical fields to the materials, thereby causing material movement through and among the channels and/or chambers, i.e., cations will move toward the negative electrode, while anions will move toward the positive electrode.

Such electrokinetic material transport and direction systems include those
20 systems that rely upon the electrophoretic mobility of charged species within the electric field applied to the structure. Such systems are more particularly referred to as electrophoretic material transport systems. Other electrokinetic material direction and transport systems rely upon the electroosmotic flow of fluid and material within a channel or chamber structure, either alone, or in conjunction with the electrophoretic forces
25 previously described, which electroosmotic flow results from the application of an electric field across such structures. In brief, when a fluid is placed into a channel which has a surface bearing charged functional groups, e.g., hydroxyl groups in etched glass channels or glass microcapillaries, those groups can ionize. In the case of hydroxyl functional groups, this ionization, e.g., at neutral pH, results in the release of protons from the surface and into
30 the fluid, producing a concentration of protons near the fluid/surface interface, and creating a positively charged sheath surrounding the bulk fluid in the channel. Application of a voltage gradient across the length of the channel, causes the proton sheath to move in the direction of the voltage drop, i.e., toward the negative electrode. Flow in the opposite

direction is achieved by either reversing the voltage gradient, or by providing a channel bearing positively charged ionizable groups, e.g., amino groups, etc.

"Controlled electrokinetic material transport and direction," as used herein, refers to electrokinetic systems as described above, which employ active control of the voltages applied at multiple, i.e., more than two, electrodes. Rephrased, such controlled electrokinetic systems concomitantly regulate voltage gradients applied across at least two intersecting channels. Controlled electrokinetic material transport is described in Published PCT Application No. WO 96/04547, to Ramsey, which is incorporated herein by reference in its entirety for all purposes. In particular, the preferred microfluidic devices and systems described herein, include a body structure which includes at least two intersecting channels or fluid conduits, e.g., interconnected, enclosed chambers, which channels include at least three unintersected termini. The intersection of two channels refers to a point at which two or more channels are in fluid communication with each other, and encompasses "T" intersections, cross intersections, "wagon wheel" intersections of multiple channels, or any other channel geometry where two or more channels are in such fluid communication. An unintersected terminus of a channel is a point at which a channel terminates not as a result of that channel's intersection with another channel, e.g., a "T" intersection. In preferred aspects, the devices will include at least three intersecting channels having at least four unintersected termini. In a basic cross channel structure, where a single horizontal channel is intersected and crossed by a single vertical channel, controlled electrokinetic material transport operates to controllably direct material flow through the intersection, by providing constraining flows from the other channels at the intersection. For example, assuming one was desirous of transporting a first material through the horizontal channel, e.g., from left to right, across the intersection with the vertical channel. Simple electrokinetic material flow of this material across the intersection could be accomplished by applying a voltage gradient across the length of the horizontal channel, i.e., applying a first voltage to the left terminus of this channel, and a second, lower voltage to the right terminus of this channel, or by allowing the right terminus to float (applying no voltage). However, this type of material flow through the intersection would result in a substantial amount of diffusion at the intersection, resulting from both the natural diffusive properties of the material being transported in the medium used, as well as convective effects at the intersection.

In controlled electrokinetic material transport, the material being transported across the intersection is constrained by low level flow from the side channels, e.g., the top and bottom channels. This is accomplished by applying a slight voltage gradient along the

path of material flow, e.g., from the top or bottom termini of the vertical channel, toward the right terminus. The result is a "pinching" of the material flow at the intersection, which prevents the diffusion of the material into the vertical channel. The pinched volume of material at the intersection may then be injected into the vertical channel by applying a voltage gradient across the length of the vertical channel, i.e., from the top terminus to the bottom terminus. In order to avoid any bleeding over of material from the horizontal channel during this injection, a low level of flow is directed back into the side channels, resulting in a "pull back" of the material from the intersection.

In addition to pinched injection schemes, controlled electrokinetic material transport is readily utilized to create virtual valves which include no mechanical or moving parts. Specifically, with reference to the cross intersection described above, flow of material from one channel segment to another, e.g., the left arm to the right arm of the horizontal channel, can be efficiently regulated, stopped and reinitiated, by a controlled flow from the vertical channel, e.g., from the bottom arm to the top arm of the vertical channel. Specifically, in the 'off' mode, the material is transported from the left arm, through the intersection and into the top arm by applying a voltage gradient across the left and top termini. A constraining flow is directed from the bottom arm to the top arm by applying a similar voltage gradient along this path (from the bottom terminus to the top terminus). Metered amounts of material are then dispensed from the left arm into the right arm of the horizontal channel by switching the applied voltage gradient from left:top, to left:right. The amount of time and the voltage gradient applied dictates the amount of material that will be dispensed in this manner.

In particularly preferred aspects, electrokinetic material transport is controlled through the application of appropriate currents through the channels of the system, in order to propagate material movement therethrough. The use of current control in electrokinetic material transport systems is described in detail in commonly owned U.S. Patent Application No. 08/678,436, filed July 3, 1996, and Published PCT Application No. 98/00707, both of which are incorporated herein by reference. In brief, in electrokinetic material transport systems, the relative potentials at the intersections of the channels dictates the direction and velocity of material movement at those intersections. Control of these potentials has typically relies upon the calculation of applied voltages based upon the desired potential at the intersections and the resistance of the channel between the intersection and the electrodes at which voltages are applied. By monitoring and controlling

the current, the potential at the intersection is maintained at the desired level, and the applied voltages are self-regulating.

Although described for the purposes of illustration with respect to a four way, cross intersections, these controlled electrokinetic material transport systems can be readily adapted for more complex interconnected channel networks, e.g., arrays of interconnected parallel channels.

A. Physical and Electrical Isolation of Reservoirs/Ports

As noted previously, in the design and fabrication of microfluidic systems, and underlying goal is to miniaturize the entire system. This is typically done either to reduce volume, increase the speed of the operation, or multiplex the particular operation, e.g., incorporate multiple operations within the same unit space occupied by the device. In accomplishing these goals, however, the channel networks that effectively define the functional space of a given microfluidic system become much smaller. As a result of smaller channel networks, or more complex networks being incorporated into the same unit space, the access points for these channel networks, e.g., reservoirs, electrical access ports and the like, are drawn closer and closer together.

As these access ports are drawn closer together, it becomes more difficult to practically isolate one port from another. For example, where the access ports are used to introduce fluids into the channel networks of the system, the closer the ports are together or the smaller they become, the more difficult it becomes to introduce fluid volumes separately into different ports. This is true for manual introduction of fluids, e.g., using a pipettor, as well as automatic methods, e.g., using robotic fluid handling systems.

In a similar problem, as access ports are placed closer together, it also becomes more difficult to isolate those ports electrically. This is of particular importance in microfluidic systems that utilize electrical systems operably coupled to the channel networks, such as in electrical sensing systems, e.g., amperometric, potentiometric, etc., and/or electrokinetic material transport that are used for transport of materials through the channel networks, as described above. In particular, where the ports of the system are used for electrical access, the possibility of bridging currents, or "shorts," between two or more adjacent or at least proximal electrodes increases, e.g., across the surface of the device as a result of fluids, dirt or oils deposited on the surface of the device.

The present invention generally addresses these problems by providing microfluidic devices that include a cover layer that provides an effective barrier between neighboring reservoirs, to prevent fluid and/or electrical links from forming between

neighboring electrodes. The barrier optionally includes a ridge around each of the reservoirs, e.g., an annular ridge surrounding a circular reservoir. The ridge has the effect of preventing fluid 'spill-over' from one well entering into another adjacent well. Similarly, the ridge effectively creates a longer path length across which any electrical bridging current, e.g., short circuit, must travel. Typically, these ridges extend at least 0.1 mm from the surface of the cover layer, preferably, at least 1 mm and in some cases, at least 2 mm or more, from the upper surface of the cover layer. In many cases, the barrier, e.g., as provided by the ridge structure, will increase the effective path length between neighboring wells by at least 1.5X, preferably at least 2X, and often at least 3-5X over that provided by the reservoirs in the body structure, alone.

The use of separate or integrated holding structures for microfluidic devices is described in commonly owned Provisional Patent Application No. 08/906,441, filed August 5, 1997, and incorporated herein by reference in its entirety for all purposes.

In addition to providing an effective barrier between neighboring reservoirs, in some cases, the upper surface comprises a hydrophobic material to prevent deposition/aggregation of fluids on that surface which might physically or electrically contaminate neighboring reservoirs. In such cases, a hydrophobic material, e.g., a polymer, is coated on the surface of the cover layer. Preferably, however, and as described in greater detail below, the cover layer itself is fabricated from a hydrophobic polymer material.

B. Increased Volume Capacity of Reservoirs

The cover layer component of the microfluidic devices of the present invention also provides the capability to increase the volume capacity of the reservoirs of those devices. In particular, the apertures disposed in the cover layer can increase the total depth of the fluid reservoirs of the device by extending those reservoirs. While fluid volume is not a critical limitation in many microfluidic applications, there are some instances where substantial variations in fluid volume from, e.g., evaporation, can have an effect on a particular operation. This is typically due to concentration of one or more solutes within the fluids, e.g., salts, enzymes, etc. By increasing the fluid volume capacity of the reservoirs, one can substantially mitigate any effects resulting from a partial evaporation of fluids by reducing the percentage of evaporation.

Typically, the apertures disposed in the cover layer add to the depth of the reservoirs in the body structure. In doing so, the apertures are typically at least 1 mm deep, preferably at least 2 mm deep, and often at least 5 mm deep. This typically results in reservoirs in the overall device, e.g., from the combination of the ports in the body structure

and the apertures in the cover layer, having volumes of at least 5 μl , preferably at least 10 μl , more preferably at least 20 μl , often at least 50 μl , and in some cases, at least 100 μl . In any event, the volume of the reservoirs of the overall device will typically fall in the range between about 1 and about 200 μl , preferably between about 2 and 100 μl , more preferably between about 5 and about 100 μl , and still more preferably, between about 5 and 50 μl .

III. Fabrication of Cover Layers

The cover layer aspect of the microfluidic devices described herein may generally be fabricated from any of a number of different materials using a number of different methods. For example, the materials and methods described above in the manufacture of the microfluidic elements of the device may also be employed in the manufacture of the cover layer. While these methods are effective, in preferred aspects, more conventional manufacturing techniques are used to produce the cover layer. In particular, because the cover layer does not need to be manufactured to the tolerances of the microfluidic elements of the devices of the invention, they can generally be manufactured using less precise and less expensive or time consuming methods and from less costly materials.

For example, in a layered microfluidic device fabricated from two glass layers, fabrication of the ports or reservoirs in one layer, e.g., by drilling or air abrasion techniques, can take a substantial amount of time. Further, the amount of time required for such fabrication increases in a non-linear, e.g., exponential, fashion with increasing substrate thickness. Conversely, reduction of substrate thickness reduces the amount of time required to fabricate the reservoirs, in an exponential fashion. Because a portion of the volume of the reservoirs in the final microfluidic device is optionally supplied by the cover layer element, the substrate layers used to fabricate the body structure of the microfluidic device can be substantially thinner. Specifically, less of the total desired volume of the reservoir is a function of substrate thickness. As a result, fabrication time and cost associated with the manufacturing of reservoirs in the body structure are substantially reduced.

Typically, the cover layer comprises an injection molded polymeric or plastic part, fabricated from any of a number of different manufacturable plastics. For example, the cover layer is typically fabricated from any of the polymeric materials described above for fabricating the body structure of the microfluidic device, e.g., polymethylmethacrylate

(PMMA), polycarbonate, polytetrafluoroethylene (TEFLON™), polyvinylchloride (PVC), polydimethylsiloxane (PDMS), polysulfone, polystyrene, polymethylpentene, polypropylene, polyethylene, polyvinylidene fluoride, ABS, and the like. In alternate aspects, the cover layer is optionally fabricated from non-polymeric materials, e.g., silica-based substrates, such as glass, quartz, silicon, as well as ceramics or metals.

Attachment of the cover layer to the body structure of the device is also typically accomplished by well known methods, including adhesive bonding, ultrasonic welding, solvent welding, thermal bonding, and the like. In preferred aspects, the cover layer is attached to the body structure of the device using an adhesive material, and more preferably, U.V. curable adhesives are used to join the cover layer with the body structure. Such adhesives are generally commercially available, e.g., from 3M Corporation. In particularly preferred aspects, the selected adhesive is electrically insulating, e.g., nonconductive, non-soluble and/or non-leaching in application buffers, low fluorescing, and the like. Further, preferred adhesives include, e.g., Norland 65 and Dymax A, commercially available from Norland Corp. (New Jersey), and Dymax Corp. (Massachusetts), respectively. In some cases, where additional long-term adhesion is desired, e.g., for storage, and where glass substrates are used, the surface of the glass may be further treated in order to provide better adhesive properties. For example, the surface of the glass substrate may be silanized using, e.g., a bifunctional silane, such as 3-aminopropyltriethoxysilane, which provides a covalent interaction between the adhesive and the glass substrate.

In alternate aspects, the body structure is attached to the cover layer via a clamping mechanism. In such aspects, an optional flexible gasket, e.g., latex, silicone, etc., is placed between the upper surface of the body structure and the lower surface of the cover layer. The body structure is then compressively clamped against the cover layer forming a sealed, joined structure. Suitable clamping mechanisms may be separate from the body structure/cover layer assembly, i.e., screw clamps, clip-style clamps, e.g., that clamp the edges of the body structure and cover layer, and the like. Alternatively, integrated clamping mechanisms are provided as a portion of the cover layer, into which the body structure is snapped. Such clamping systems are described in greater detail below, with reference to Figure 3B.

IV. Illustrated Embodiment

One aspect of the cover layer used in conjunction with the microfluidic devices of the present invention is shown in Figure 2, from the top (Fig. 2A), side (Fig. 2B), bottom (Fig. 2C), top perspective (Fig. 2D) and bottom perspective views (Fig. 2E). As shown, the cover layer 200 is planar in shape having an upper planar surface 202 and a lower planar surface 204. Also included are a plurality of apertures 206 disposed through the cover layer, e.g., from the upper to lower planar surfaces. Apertures 206 are positioned within the cover so as to align with ports/reservoirs in the body structure of a microfluidic device (e.g., as shown in Figure 1) when that body structure is mated to the lower planar surface 204 of cover layer 200.

As shown, the apertures 206 in cover layer 200 are provided in a gridded pattern to match a similar gridded pattern of ports on the body structure of the device. Typically, the gridded arrangement of apertures and ports (collectively, reservoirs) are positioned on regular centers, e.g., 9 mm, 4.5 mm etc., to match the spacing of typical multi-well plates, e.g., 96-well, 384-well, 1536-well, etc.

As shown, an annular ridge 208 is provided on the upper surface 202 of the cover layer 200, surrounding each separate aperture 206. This ridge provides a barrier between neighboring reservoirs in the overall device and also functions to increase the effective volume of each reservoir in the resulting device. In addition, the apertures 206 in the cover layer are optionally provided with tapered walls 210, which are wider at the upper surface and narrower at the lower surface. The tapered walls allow the apertures to perform a funnel-like function, in the introduction of fluids into the ports of the microfluidic devices. Specifically, wider openings facilitate introduction of fluids into the reservoir.

Also as shown, the lower planar surface 204 of the cover layer 200, has fabricated thereon, a series of raised ridges 212, which function as alignment structures to ensure the body structure of the microfluidic device 100 (from Figure 1), is properly aligned with the cover layer during the bonding or mating process. Although illustrated as ridges, it will be understood that a number of different alignment structures may be provided upon the lower planar surface for aligning the body structure of the device with the cover layer. For example, a recessed region, which is configured to fit the body structure may be used, whereby placement of the body structure into the recessed region positions the body structure to be appropriately aligned with the apertures in the cover layer. Alternatively, alignment pins may be provided extending from the lower surface, against which the body structure may rest, when appropriately aligned with the cover layer.

Also, included on the lower surface 204 of the cover layer 200 are small high spots 214. These high spots, or bumps, maintain the body structure in a position slightly set off of the lower surface 204 when the body structure is mated with the cover layer. The small set off resulting from high spots 214 allows a bonding adhesive material to wick into the space between the body structure and the cover layer for attaching the body structure to the cover layer.

As shown, the cover layer 200 includes side-walls 216, which extend from the lower planar surface 204, effectively creating a hollow-backed structure. This hollow-backed structure permits the mounting of a body structure of a microfluidic device to the lower surface of the cover layer without altering the overall profile of the cover layer, e.g., permitting the combined device-cover layer to be laid flat upon a surface or stacked with other like devices, as well as providing benefits in manufacturing, e.g., curing/hardening of molded parts, etc.

In addition to providing alignment structures for mounting a body structure to the cover layer, as shown, the cover layer also includes additional alignment structures 218 and 220. These alignment structures permit the appropriate alignment of the overall device into an appropriate base unit, such as a controller/detector instrument (not shown). In particular, alignment holes 218 provided disposed through the cover layer are complementary to alignment pins that are provided on a controller/detector instrument (not shown). By matching the pins of the controller/detector instrument with the holes on the overall device, one is assured of proper alignment of the device with the appropriate elements of the instrument, e.g., electrodes, optical detectors, thermal blocks, etc. In addition to alignment holes 218, the cover layer 200 also includes a beveled corner 220, which further ensures proper alignment of the device in the controller/detector instrument. Again, a number of different types of alignment structures may be used to accomplish this same purpose, including irregular edges, e.g., beveled, tabbed, etc., alignment pins, non-uniform shapes and the like.

As shown in Figure 2A, the cover layer also includes convenience features. For example, textured regions 222 are provided on side-walls 216, to provide gripping surfaces for manual handling of the cover layer and assembled device. Also provided is registry port 224 disposed through the cover layer. Different numbers, sizes and/or shapes of registry ports are optionally provided in the cover layer to register the type of microfluidic device that has been inserted in a controller/detector instrument. This ensures that the proper interface is used, and/or the proper control program is being run.

Figure 3A illustrates the fully assembled microfluidic device 300 including the body structure 100 mated with the lower surface of the cover layer 200, and bonded using, e.g., an adhesive, as described above. The dimensions of planar devices, e.g., as shown in Figure 3A, can vary substantially depending upon the application for which the device is to be used. Typically, however, the fully assembled devices have a rectangular shape and range from about 5 mm to about 200 mm on a side, and preferably are in the range of from about 10 mm to about 100 mm, and still more preferably, in the range of from about 20 mm to about 70 mm, e.g., about 50 mm on a side. For example, a square device approximately 50 mm on a side is shown. Such devices provide ease of handling as well as ready access to equipment already sized for handling substrates of this size, i.e., photographic slides.

Figure 3B illustrates a clamping mechanism integrated into the cover layer of the device. In particular, as shown, the cover layer 200 (partially shown), includes on its bottom surface, clip tabs 310. These clips flex to allow insertion of the body structure 100, then snap into place to lock the body structure 100 in position against the cover layer 200 with barbs 312. Gasket 314 provides a seal between the two structures, as well as providing the necessary flexibility to permit the clips to compressively clamp the body structure against the cover layer. The gasket 314 is optionally fabricated from a flexible material, such as latex, silicone, or the like, or a semi-rigid material, such as polytetrafluoroethylene (Teflon™), polypropylene, etc.

Although illustrated as a planar cover layer attached to a planar body structure, it will be appreciated that an appropriate cover layer is optionally joined to a non-planar microfluidic system, e.g., a tubular capillary or the like. In such cases, apertures in the cover layer are again fabricated to align with the ports, e.g., inlets and outlets of the capillary channel.

In addition to the above-noted advantages, e.g., isolation of reservoirs, increased reservoir volume, etc., the cover layers described for use in conjunction with the present invention also optionally include other useful features. For example, the shape of the aperture in the cover layer is optionally configured to receive a complementary structure on a filling apparatus, e.g., syringe or pump. Specifically, in some cases it is desirable to use a positive pressure source to assist in filling the channel networks of a microfluidic device with fluid. This is typically useful where the filling solution, e.g., running buffer, separation matrix, etc., is slower at wicking into the channel network via capillary action, due to viscosity effects. In operation, the running buffer, separation matrix, etc. is placed

into one reservoir of the microfluidic device. A positive pressure is then applied to that reservoir thereby forcing the fluid throughout the channel network.

Application of the positive pressure is preferably carried out using an apparatus that sealably fits over the reservoir while not actually contacting the fluid contained therein. Examples of such devices, as well as appropriately configured apertures on the cover layer, are illustrated in Figure 2F. As shown, the filling device 250 includes a syringe 252 and a rigid tube 254, e.g., a needle. The rigid tube/needle is inserted through a rubber, e.g., silicone, latex, etc., ball stopper 256 that is selected to properly fit within the aperture 206 in the cover layer 200. The conical shape of aperture 206 permits the ball stopper 256 to be inserted into the aperture 206. Compression of the stopper against the walls of the aperture then creates a positive seal. The rigid tube/needle 254 is further positioned within the ball stopper 256 so as to be able to apply pressure to the reservoir 106, without contacting the fluid within the reservoir 106. In particular, the tube is inserted through the stopper such that little or no tube length extends beyond the surface of the stopper, e.g., less than 2 mm, preferably less than 1 mm and more preferably less than 0.5 mm of the tube extending beyond the surface of the stopper.

Application of pressure by activation of syringe 252, then forces fluid within reservoir 106 into the channel network (not shown) of the device 100. Alternatively shaped stoppers 258 and 260 are also shown for use in the filling device. In the preferred stopper 260, the ball portion 262 of the stopper inserts into the aperture, and compression of the ball portion provides a positive seal. The ball shape allows one to insert the filling device at an angle of up to approximately 15° from normal to the plane of the cover layer 200, without adversely effecting the sealing ability of the filling device. Alternatively, the ball can be inserted until the flat ledge 264 contacts the upper surface of the cover surrounding the aperture 106. This provides a secondary seal for the filling device, in addition to the ball stopper. Although illustrated as a syringe, it will be appreciated that virtually any source of pressure is suitable for use in the filling device, including external pumps, pipettors and the like. The stopper optionally includes a recessed region 266 at the top for receiving the syringe 252 or pump outlet.

Additional functions are also optionally performed by the cover layer. For example, in some cases, it may be desirable to perform a separation function, e.g., a filtration, cell separation, molecular weight separation, affinity, charge-based or hydrophobic interaction type separations, on a sample that is to be introduced into the microfluidic device. Accordingly, an appropriate filtration or separation medium or

membrane is optionally provided within or across the aperture on the cover layer. When the cover layer is mated to the body structure of the device, introduction of a sample into the body structure requires passage through the filter or membrane, and separation of particulate components, high molecular weight materials, and the like.

5 In further aspects, the cover layer performs a fluid handling and direction function, e.g., a manifolding function, where an aperture in the cover layer communicates with more than one reservoir on the body structure of the device, e.g., 2, 3, 4, 5, 10 or even 20 different reservoirs. Such a system is particularly useful where a single sample is to be subject to multiple different analyses within the body structure of the microfluidic device, 10 e.g., in diagnostic applications where a single patient sample may be subject to multiple diagnostic tests. A variety of other modifications will be apparent to one of ordinary skill in the art, and are generally encompassed by the present invention, as set forth in the appended claims.

Alternatively, the cover layer may include other components useful in the 15 operation of the microfluidic device and system, including e.g., integrated optical elements, e.g., lenses, gratings, coatings, polished detection windows, etc., as described in commonly owned U.S. Patent Application No. 09/030,535, entitled "Microfluidic Devices and Systems Incorporating Integrated Optical Elements," filed of even date (Attorney Docket No. 100/03500), which is incorporated herein by reference, in its entirety for all purposes. Such 20 elements supplement or replace optical elements from an external detection system.

III. System Description

As alluded to above, the microfluidic devices described herein, are generally operated in conjunction with a controller instrument. Typical controller instruments include 25 material transport systems, for affecting material, e.g., fluid, movement within and among the channels and chambers of a microfluidic device. For example, in the case of microfluidic systems employing pressure based fluid flow or that incorporate pressure actuated micropumps and valves, the controller instrument typically includes pressure sources as well as appropriate manifolds for delivering the appropriate pressures to 30 complementary ports on the microfluidic device. The instrument then applies pressure/vacuum to activate the pumps and valves, or directly to fluids, to move those fluids through the channels of the device in a controlled fashion. In the case of microfluidic systems employing electrokinetic material transport systems, the controller typically includes an electrical power supply that is capable of delivering voltage gradients across the

length of channels within the microfluidic device, as described above, when the device is mounted in the controller. Examples of particularly preferred power supplies are described in, e.g., Published International Application No. WO 98/00707, which is incorporated herein by reference in its entirety for all purposes.

5 As such, the controller typically includes an appropriate interface for delivering the voltage gradients to the channels of the device. Such interfaces are generally described in detail in commonly owned U.S. Patent Application No. 08/919,707, filed October 9, 1997, and incorporated herein by reference for all purposes. In brief, such interfaces typically include a number of electrodes, operably coupled to electrical leads
10 from the power supply. The controller also typically includes a nesting region, e.g., a well or platform, upon which the microfluidic device is mounted. The electrodes are positioned so as to be placed into electrical contact with the channels of the device. In preferred aspects, this is accomplished by providing a "clam shell" lid hinged to the nesting region so as to close over the top of the device. The device, e.g., as shown in Figures 1-3, is mounted
15 on the nesting region with the reservoirs facing upward. The electrodes protruding from the lower surface of the clam-shell lid then insert into the reservoirs on the upper surface of the microfluidic device when the clam-shell lid is closed, so as to be placed into electrical contact with fluids in those reservoirs.

 Environmental control elements are optionally included in the controller
20 instrument, e.g., for maintaining the environmental conditions to which the microfluidic device is exposed, at optimal levels. For example, the controller optionally includes a thermal control element, e.g., a heating block, peltier device, etc.

 In addition to including control elements, in preferred aspects, the controller instrument also includes a detection system for detecting the results of an operation
25 performed in the microfluidic device. As such, the instrument is also referred to as a controller/detector instrument.

 Examples of particularly preferred detection systems include fluorescent detection systems. Typically, these detection systems include a light source, such as a laser, laser diode, LED or high intensity lamp, within the controller/detector instrument. The
30 detection system also typically includes appropriate optics, e.g., lenses, beam splitters, filters, dichroics, and the like, for directing the light source at the detection window of a microfluidic device mounted on the controller/detector. The optics also gather emitted fluorescence emanating from the channel(s) of the device, separate out reflected excitation light, and detect the emitted fluorescence, e.g., using a photodiode or photomultiplier tube

(PMT). Other optical detection systems are optionally included within the controller/detector instrument, e.g., absorbance or colorimetric detection systems, and the like. Both fluorescence based and absorbance based detection systems are well known in the art.

5 The controller/detector instrument is also typically interfaced with an appropriate processor, e.g., an appropriately programmed computer, which instructs the operation of the material transport system, e.g., applied voltages, timing, etc. The processor is also typically is operably linked to the detection system of the controller/detector instrument, so that the computer can receive, store and manipulate the data collected from the detection system.

10 In general, the invention provides for the use of the microfluidic devices and systems herein, for example, for fluid movement within the device or for practicing an assay with the device, e.g., as set forth above. Use of the various components of the devices and systems set forth herein to make the devices and systems of the invention are also provided.

15 All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be

20 practiced within the scope of the appended claims:

WHAT IS CLAIMED IS:

1. A microfluidic device, comprising:
 - a body structure comprising at least a first microscale channel network
5 disposed therein, the body structure having a plurality of ports disposed in the body structure, each port being in fluid communication with one or more channels in the first channel network; and
 - a cover layer comprising a plurality of apertures disposed through the cover layer, the cover layer being mated with the body structure whereby each of the apertures is
10 aligned with at least one of the plurality of ports.
2. The microfluidic device of claim 1, wherein each of the body structure and the cover layer separately comprises at least a first surface, the plurality of ports being disposed in the first surface of the body structure, and the plurality of apertures being
15 disposed in the first surface of the cover layer, the first surface of the cover layer being mated to the first surface of the body structure such that the apertures align with and are in fluid communication with the ports.
3. The microfluidic device of claim 1, wherein the first surface of the body
20 structure and the first surface of the cover layer are planar.
4. The microfluidic device of claim 1, wherein the cover layer further comprises:
 - a second surface opposite the first surface, the apertures extending from the
25 first surface to the second surface; and
 - a plurality of raised annular ridges disposed on the second surface, the ridges surrounding each of the apertures.
5. The microfluidic device of claim 4, wherein the ridges are raised at least 0.5
30 mm relative to the second surface of the cover layer.
6. The microfluidic device of claim 4, wherein the ridges are raised at least 1 mm relative to the second surface of the cover layer.

7. The microfluidic device of claim 4, wherein the ridges are raised at least 2 mm relative to the second surface of the cover layer.

5 8. The microfluidic device of claim 1, wherein the apertures are at least 1 mm deep.

9. The microfluidic device of claim 1, wherein the apertures are at least 2 mm deep.

10 10. The microfluidic device of claim 1, wherein the apertures are at least 5 mm deep.

11. The microfluidic device of claim 4, wherein the second surface of the cover layer comprises a hydrophobic polymer.

15 12. The microfluidic device of claim 11, wherein the hydrophobic polymer comprises polytetrafluoroethylene.

20 13. The microfluidic device of claim 1, wherein alignment of the apertures with the wells results in a plurality of reservoirs, each of the reservoirs having a volume of at least 10 μ l.

25 14. The microfluidic device of claim 1, wherein alignment of the apertures with the wells results in a plurality of reservoirs, each of the reservoirs having a volume of at least 20 μ l.

30 15. The microfluidic device of claim 1, wherein alignment of the apertures with the wells results in a plurality of reservoirs, each of the reservoirs having a volume of at least 50 μ l.

16. The microfluidic device of claim 1, wherein alignment of the apertures with the wells results in a plurality of reservoirs, each of the reservoirs having a volume of at least 100 μ l.

17. The microfluidic device of claim 1, wherein alignment of the apertures with the wells results in a plurality of reservoirs, each of the reservoirs having a volume of between about 5 and about 200 μ l.

5

18. The microfluidic device of claim 1, wherein alignment of the apertures with the wells results in a plurality of reservoirs, each of the reservoirs having a volume of between about 10 and about 100 μ l.

10

19. The microfluidic device of claim 1, wherein the cover layer is bonded to the first surface of the body structure with an adhesive.

20. The microfluidic device of claim 19, wherein the adhesive is a UV curable adhesive.

15

21. The microfluidic device of claim 1, wherein the cover layer is clamped to the first surface of the body structure.

22. The microfluidic device of claim 21, further comprising a gasket disposed between the cover layer and the first surface of the body structure.

20

23. The microfluidic device of claim 1, wherein the cover layer further comprises at least a first alignment structure for aligning the body structure on the first surface of the cover layer.

25

24. The microfluidic device of claim 23, wherein the first alignment structure comprises one or more raised ridges disposed around a perimeter of the plurality of apertures.

30

25. The microfluidic device of claim 23, wherein the first alignment structure comprises a recessed portion of the first surface of the cover layer.

26. The microfluidic device of claim 23, wherein the first alignment structure comprises a plurality of alignment pins extending from the first surface of the cover layer.

27. The microfluidic device of claim 23, wherein the cover layer comprises at least a second alignment structure complementary to an alignment structure on a controller/detector apparatus, to align the microfluidic device in the controller/detector apparatus.

28. The microfluidic device of claim 27, wherein the second alignment structure comprises one or more alignment holes, and the complementary alignment structure comprises one or more alignment pins.

29. The microfluidic device of claim 27, wherein the second alignment structure comprises an irregular edge of the cover layer.

30. The microfluidic device of claim 27, wherein the irregular edge comprises a beveled corner of the cover layer.

31. The microfluidic device of claim 1, wherein the apertures are disposed in the cover layer in a grid pattern on approximately 9 mm centers.

32. The microfluidic device of claim 1, wherein the apertures are disposed in the cover layer in a grid pattern on approximately 4.5 mm centers.

33. The microfluidic device of claim 1, wherein the cover layer is fabricated from a polymeric material.

34. The microfluidic device of claim 33, wherein the cover layer is an injection molded polymeric part.

35. The microfluidic device of claim 33, wherein the cover layer is a cast polymer part.

36. The microfluidic device of claim 1, wherein the body structure comprises: a first planar substrate having at least a first surface, at least a first groove fabricated into the first surface of the first substrate; and

a second planar substrate overlaying and bonded to the first planar substrate, sealing the groove to form the at least first channel network, the second planar substrate including a plurality of ports disposed therethrough, the ports communicating with the at least first channel network.

5

37. The microfluidic device of claim 36, wherein the first and second planar substrates comprise silica based substrates.

38. The microfluidic device of claim 37, wherein the silica based substrate is glass.

10

39. The microfluidic device of claim 36, wherein the first and second substrates comprise polymeric substrates.

40. The microfluidic device of claim 39, wherein the polymeric substrate is selected from polymethylmethacrylate (PMMA), polycarbonate, polytetrafluoroethylene, polyvinylchloride (PVC), polydimethylsiloxane (PDMS), polysulfone, polystyrene, polymethylpentene, polypropylene, polyethylene, polyvinylidene fluoride and ABS.

15

41. The microfluidic device of claim 1, wherein the microfluidic device/cover layer comprises a rectangle measuring approximately 50 mm on at least one edge.

20

42. The microfluidic device of claim 41, wherein the microfluidic device comprises a square shape measuring approximately 50 mm X 50 mm.

25

43. A microfluidic device, comprising:

a body structure comprising at least a first channel network disposed therein, and a plurality of reservoirs disposed at and in fluid communication with different points of the channel network;

30

a cover layer comprising a plurality of apertures disposed therethrough each aperture forming at least a portion of a separate one of the plurality of reservoirs, wherein the cover layer further comprises a plurality of raised ridges extending from and surrounding an outer edge of each of the apertures.

44. A microfluidic system, comprising:
a microfluidic device of claim 1; and
a controller/detector apparatus configured to receive the microfluidic device,
the controller/detector apparatus comprising an optical detection system and a material
5 transport system, the detection system and transport system being operably interfaced with
the microfluidic device.

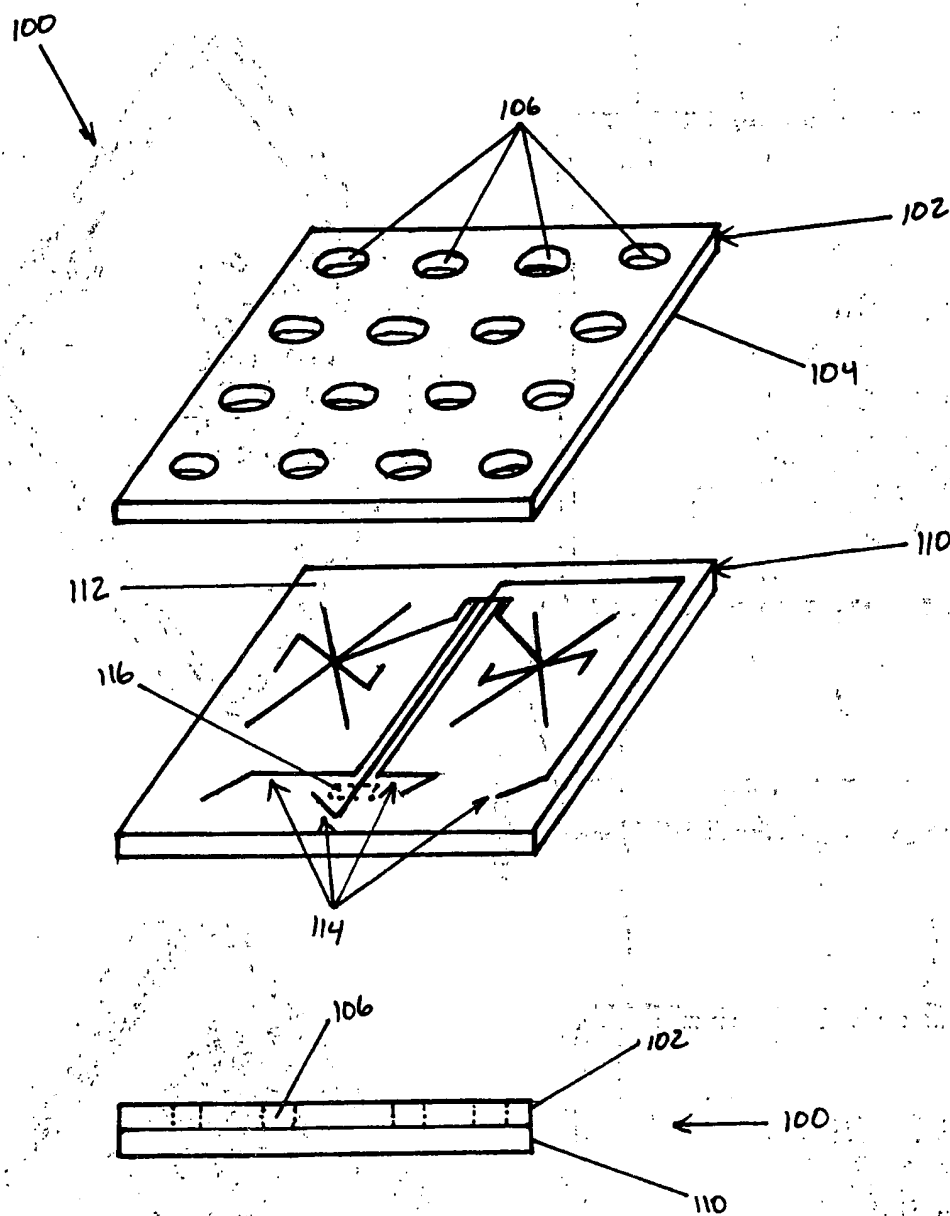


Fig. 1.

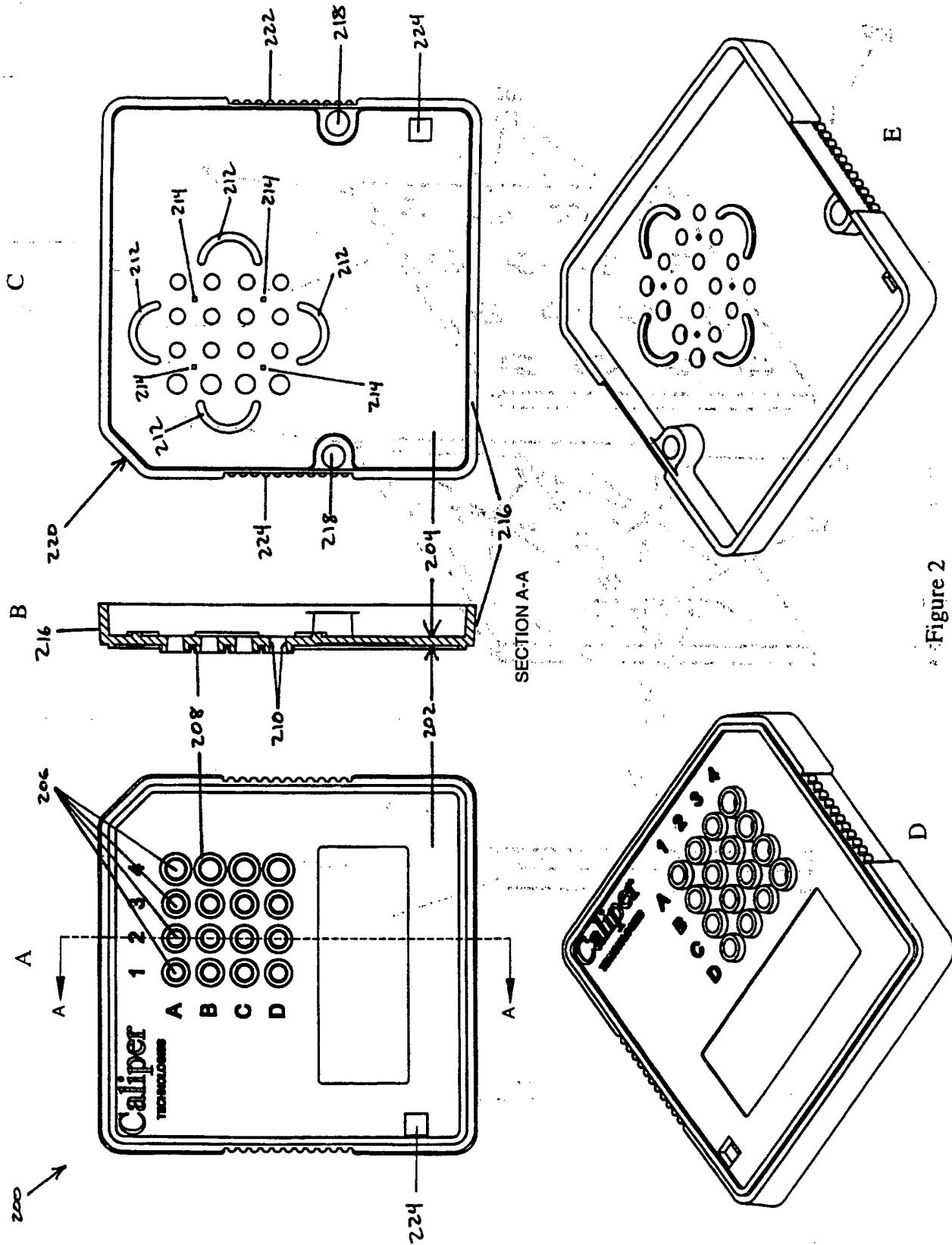


Figure 2

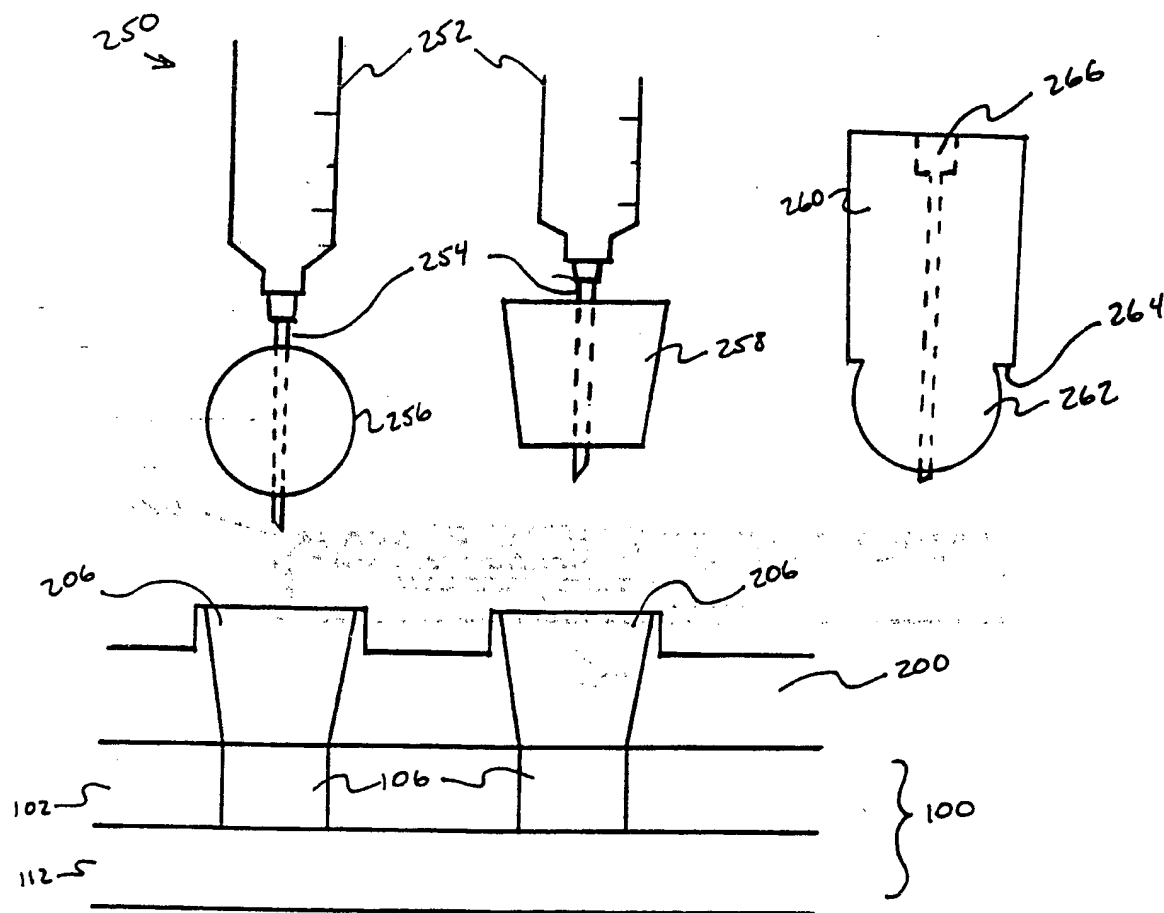


Fig. 2F

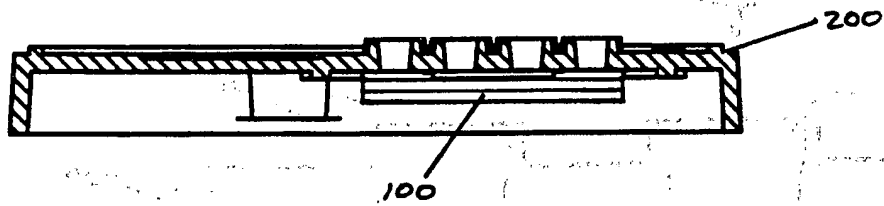


Fig. 3A

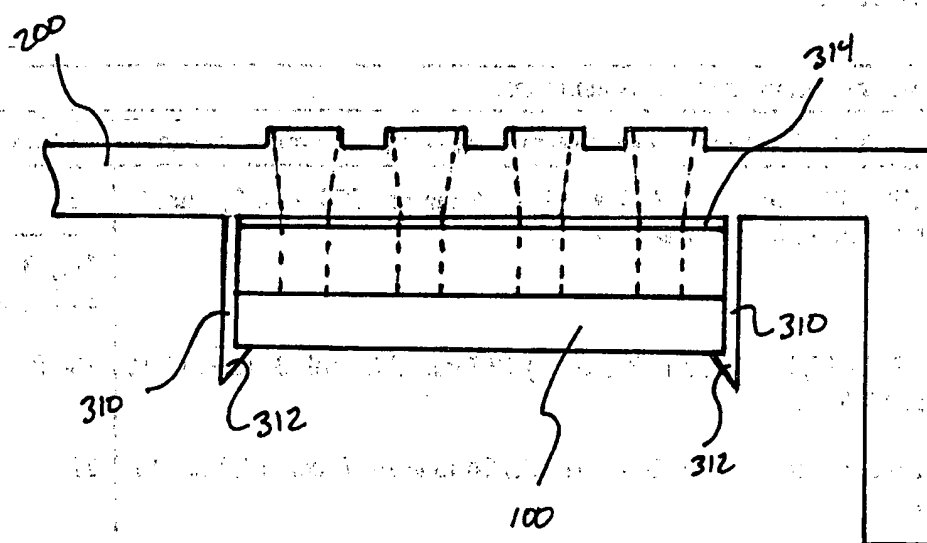


Fig. 3B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/03902

A. CLASSIFICATION OF SUBJECT MATTER IPC(6) :B01L 3/00; G01N 27/26, 27/447 US CL :422/50, 99, 102, 129; 204/600, 601 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 422/50, 99, 102, 129; 204/600, 601 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Consulted with Examiner Noguerola about a search relevant to microfluidic devices. Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Please See Extra Sheet.		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,E — Y,E	US 5,882,465 A (McREYNOLDS) 16 March 1999, col. 3, lines 3-67, col. 4, lines 1-4 and 55-60.	36 1-3, 8-12, 19-20, 31-35, 37-44
Y	US 5,427,946 A (KRICKA et al.) 27 June 1995, col. 3, lines 15-18 and 37-56.	13-18
Y	US 5,640,995 A (PACKARD et al.) 24 June 1997, col. 10, lines 10-13.	22
Y	US 5,699,157 A (PARCE et al.) 16 December 1997, abstract.	44
A	US 5,498,392 A (WILDING et al.) 12 March 1996.	
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "B" earlier document published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "A" document member of the same patent family	
Date of the actual completion of the international search 03 MAY 1999		Date of mailing of the international search report 26 MAY 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer Kevin P. Cannell Telephone No. (703) 308-0661

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/03902

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,E	US 5,885,470 (PARCE et al.) 23 March 1999.	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/03902

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS search terms: (microfluidic or micro fluidic)(3a)(device or apparatus)

(microfluidic or micro fluidic)/ti

e (Manz or Zanzucchi or Bard)/in

THE CANAL ZONE

$$E_{\text{eff}} = E_0 \left(1 - \frac{1}{2} \frac{E_0}{E_c} \right) \quad (1)$$
[illegible][illegible]

1. The first step in the process is to identify the problem or issue that needs to be addressed. This involves gathering information and understanding the context of the problem.

For the purpose of this study, the following hypotheses were formulated:

[illegible]

1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017, 2018, 2019, 2020, 2021, 2022, 2023, 2024, 2025, 2026, 2027, 2028, 2029, 2030, 2031, 2032, 2033, 2034, 2035, 2036, 2037, 2038, 2039, 2040, 2041, 2042, 2043, 2044, 2045, 2046, 2047, 2048, 2049, 2050, 2051, 2052, 2053, 2054, 2055, 2056, 2057, 2058, 2059, 2060, 2061, 2062, 2063, 2064, 2065, 2066, 2067, 2068, 2069, 2070, 2071, 2072, 2073, 2074, 2075, 2076, 2077, 2078, 2079, 2080, 2081, 2082, 2083, 2084, 2085, 2086, 2087, 2088, 2089, 2090, 2091, 2092, 2093, 2094, 2095, 2096, 2097, 2098, 2099, 2100, 2101, 2102, 2103, 2104, 2105, 2106, 2107, 2108, 2109, 2110, 2111, 2112, 2113, 2114, 2115, 2116, 2117, 2118, 2119, 2120, 2121, 2122, 2123, 2124, 2125, 2126, 2127, 2128, 2129, 2130, 2131, 2132, 2133, 2134, 2135, 2136, 2137, 2138, 2139, 2140, 2141, 2142, 2143, 2144, 2145, 2146, 2147, 2148, 2149, 2150, 2151, 2152, 2153, 2154, 2155, 2156, 2157, 2158, 2159, 2160, 2161, 2162, 2163, 2164, 2165, 2166, 2167, 2168, 2169, 2170, 2171, 2172, 2173, 2174, 2175, 2176, 2177, 2178, 2179, 2180, 2181, 2182, 2183, 2184, 2185, 2186, 2187, 2188, 2189, 2190, 2191, 2192, 2193, 2194, 2195, 2196, 2197, 2198, 2199, 2200, 2201, 2202, 2203, 2204, 2205, 2206, 2207, 2208, 2209, 2210, 2211, 2212, 2213, 2214, 2215, 2216, 2217, 2218, 2219, 2220, 2221, 2222, 2223, 2224, 2225, 2226, 2227, 2228, 2229, 2230, 2231, 2232, 2233, 2234, 2235, 2236, 2237, 2238, 2239, 2240, 2241, 2242, 2243, 2244, 2245, 2246, 2247, 2248, 2249, 2250, 2251, 2252, 2253, 2254, 2255, 2256, 2257, 2258, 2259, 2260, 2261, 2262, 2263, 2264, 2265, 2266, 2267, 2268, 2269, 2270, 2271, 2272, 2273, 2274, 2275, 2276, 2277, 2278, 2279, 2280, 2281, 2282, 2283, 2284, 2285, 2286, 2287, 2288, 2289, 2290, 2291, 2292, 2293, 2294, 2295, 2296, 2297, 2298, 2299, 2300, 2301, 2302, 2303, 2304, 2305, 2306, 2307, 2308, 2309, 2310, 2311, 2312, 2313, 2314, 2315, 2316, 2317, 2318, 2319, 2320, 2321, 2322, 2323, 2324, 2325, 2326, 2327, 2328, 2329, 2330, 2331, 2332, 2333, 2334, 2335, 2336, 2337, 2338, 2339, 2340, 2341, 2342, 2343, 2344, 2345, 2346, 2347, 2348, 2349, 2350, 2351, 2352, 2353, 2354, 2355, 2356, 2357, 2358, 2359, 2360, 2361, 2362, 2363, 2364, 2365, 2366, 2367, 2368, 2369, 2370, 2371, 2372, 2373, 2374, 2375, 2376, 2377, 2378, 2379, 2380, 2381, 2382, 2383, 2384, 2385, 2386, 2387, 2388, 2389, 2390, 2391, 2392, 2393, 2394, 2395, 2396, 2397, 2398, 2399, 2400, 2401, 2402, 2403, 2404, 2405, 2406, 2407, 2408, 2409, 2410, 2411, 2412, 2413, 2414, 2415, 2416, 2417, 2418, 2419, 2420, 2421, 2422, 2423, 2424, 2425, 2426, 2427, 2428, 2429, 2430, 2431, 2432, 2433, 2434, 2435, 2436, 2437, 2438, 2439, 2440, 2441, 2442, 2443, 2444, 2445, 2446, 2447, 2448, 2449, 2450, 2451, 2452, 2453, 2454, 2455, 2456, 2457, 2458, 2459, 2460, 2461, 2462, 2463, 2464, 2465, 2466, 2467, 2468, 2469, 2470, 2471, 2472, 2473, 2474, 2475, 2476, 2477, 2478, 2479, 2480, 2481, 2482, 2483, 2484, 2485, 2486, 2487, 2488, 2489, 2490, 2491, 2492, 2493, 2494, 2495, 2496, 2497, 2498, 2499, 2500, 2501, 2502, 2503, 2504, 2505, 2506, 2507, 2508, 2509, 2510, 2511, 2512, 2513, 2514, 2515, 2516, 2517, 2518, 2519, 2520, 2521, 2522, 2523, 2524, 2525, 2526, 2527, 2528, 2529, 2530, 2531, 2532, 2533, 2534, 2535, 2536, 2537, 2538, 2539, 2540, 2541, 2542, 2543, 2544, 2545, 2546, 2547, 2548, 2549, 2550, 2551, 2552, 2553, 2554, 2555, 2556, 2557, 2558, 2559, 2560, 2561, 2562, 2563, 2564, 2565, 2566, 2567, 2568, 2569, 2570, 2571, 2572, 2573, 2574, 2575, 2576, 2577, 2578, 2579, 2580, 2581, 2582, 2583, 2584, 2585, 2586, 2587, 2588, 2589, 2590, 2591, 2592, 2593, 2594, 2595, 2596, 2597, 2598, 2599, 2600, 2601, 2602, 2603, 2604, 2605, 2606, 2607, 2608, 2609, 2610, 2611, 2612, 2613, 2614, 2615, 2616, 2617, 2618, 2619, 2620, 2621, 2622, 2623, 2624, 2625, 2626, 2627, 2628, 2629, 2630, 2631, 2632, 2633, 2634, 2635, 2636, 2637, 2638, 2639, 2640, 2641, 2642, 2643, 2644, 2645, 2646, 2647, 2648, 2649, 2650, 2651, 2652, 2653, 2654, 2655, 2656, 2657, 2658, 2659, 2660, 2661, 2662, 2663, 2664, 2665, 2666, 2667, 2668, 2669, 2670, 2671, 2672, 2673, 2674, 2675, 2676, 2677, 2678, 26

Figure 1. The effect of the concentration of the *Agrobacterium* suspension on the transformation efficiency of *Agrobacterium* strains. The *Agrobacterium* strains were grown in the YEA medium for 24 h at 28 °C. The cell concentration of the strains was adjusted to 1.0 × 10⁸ cells/ml. The cell suspension was mixed with the plant tissue and the transformation efficiency was determined. The results were expressed as the mean ± SD of three independent experiments. The asterisks indicate the significant difference between the strains at the same concentration of the cell suspension.

the 1990s, the number of people in the world who are under 15 years of age is expected to increase from 1.1 billion to 1.5 billion. The number of people aged 65 and over is expected to increase from 200 million to 400 million. The number of people aged 15 and over is expected to increase from 3.5 billion to 4.5 billion. The number of people aged 15 and over is expected to increase from 3.5 billion to 4.5 billion. The number of people aged 15 and over is expected to increase from 3.5 billion to 4.5 billion.

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